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Functional Analysis of HIV-1 Vpr: Identification of Determinants Essential for Subcellular Localization

S. MAHALINGAM,* RONALD G. COLLMAN,†,[1] MAMATA PATEL,* CLAUDE E. MONKEN,* and A. SRINIVASAN*,‡,[1]

*Department of Microbiology and Immunology, Jefferson Cancer Institute, and ‡Institute of Biotechnology and Advanced Molecular Medicine, Thomas Jefferson University, 1020 Locust Street, Philadelphia, Pennsylvania 19107; and †Pulmonary and Critical Care Division, Department of Medicine, University of Pennsylvania School of Medicine, 522 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, Pennsylvania 19104

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Vpr is a conserved HIV-1 auxiliary protein that localizes to the nuclear region of cells. Vpr is also present in virions, and it is directed into the assembling virus when coexpressed with Gag. Each of these two localization activities may be important for Vpr function, and we recently identified regions of Vpr that are critical for virion incorporation. In this study we analyzed the Vpr domains involved in subcellular localization. Immunofluorescence staining of transfected cells showed that wild-type Vpr localized exclusively to the nuclear region. Mutations in the N-terminal domain that were designed to disrupt a predicted α-helical structure resulted in aberrant localization, while conservative substitutions showed a wild-type pattern. A region in the central portion of the protein also has the potential for helical structure, and mutagenesis of two conserved amino acids in this domain (A59, H71) impaired localization, while substitution of a third (Q65) did not. In contrast, neither the conserved Gly and Cys at positions 75–76 nor the C-terminal basic residues (R87, K95) were necessary for nuclear localization. In addition, two-residue insertions within and between the two putative helices disrupted localization but insertion in the C-terminal region did not. Thus, Vpr's subcellular localization function depends on the two putative helical domains but is independent of the conserved Gly–Cys motif and of specific C-terminal basic residues.

INTRODUCTION

HIV-1 *vpr* encodes a 14-kDa, 96-amino-acid protein that is dependent on Rev for expression (Garrett *et al.*, 1991) and is packaged into virion particles (Cohen *et al.*, 1990a; Yuan *et al.*, 1990). In addition to HIV-1, *vpr* is present in HIV-2 and most simian immunodeficiency virus (SIV) strains (Tristem *et al.*, 1992). It has significant sequence and structural homology with the *vpx* gene of HIV-2 and SIV, and it is thought that *vpx* may have originated from a duplication of *vpr* (Tristem *et al.*, 1992). However, the essential *in vivo* function(s) for which *vpr* is conserved among immunodeficiency viruses is unknown. We and others have shown that it is necessary for maximal viral replication in primary macrophages (Balliet *et al.*, 1994; Westervelt *et al.*, 1992; Balotta *et al.*, 1993; Hattori *et al.*, 1990; Connor *et al.*, 1995), which suggests that its role may be related to macrophage-dependent steps in pathogenesis, but the mechanism(s) by which Vpr enhances virus replication in macrophages or other cell types is uncertain. Vpr can modestly transactivate the HIV-1 LTR (Cohen *et al.*, 1990b) and thus may upregulate viral gene expression in newly infected cells before the appearance of Tat, and it has been found to enhance nuclear migration of the preintegration complex in newly infected nondividing cells (Heinzinger *et al.*, 1994). Alternatively, Vpr can induce cellular differentiation, which suggests that it may act by indirect effects on cellular functions (Levy *et al.*, 1993), and was recently found to block cell cycling in G2 and inhibit the development of chronically infected cells (Rogel *et al.*, 1995). In addition, soluble exogenous Vpr has been reported to upregulate virus replication in a variety of productive and nonproductively infected cells (Levy *et al.*, 1994).

Recent studies showed that Vpr localizes to the nucleus (Lu *et al.*, 1993; Zhao *et al.*, 1994a), a property that it is consistent with its nuclear migration, transactivation, cellular differentiation, and cell cycle effects. When expressed in the presence of core proteins, however, Vpr is incorporated into Gag-directed virus-like particles. This interaction is dependent on the p6 domain of Gag (Lavallee *et al.*, 1994; Lu *et al.*, 1993; Paxton *et al.*, 1993) and is responsible for its efficient packaging into virions and consequent delivery to target cells. Each of these localization functions, virion association and nuclear localization, is therefore likely to be important for Vpr's mechanism of action.

Sequence comparison and structural analysis of Vpr reveals a number of conserved amino acids (Tristem *et al.*, 1992) and predicted structural motifs. Residues 17–34 are strongly predicted to form an amphipathic α-helix by Chou–Fasman analysis (Mahalingam *et al.*, 1995b). A second region (residues 46–74) is also predicted to have helical structure (unpublished observations). We recently reported that Vpr incorporation into Gag-directed

[1] To whom correspondence and reprint requests should be addressed.

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particles depends on the N-terminal putative α-helix (Mahalingam *et al.*, 1995a,b). The goal of this study was to analyze the domains of Vpr that are involved in its subcellular localization function. Our results demonstrate critical functions for the N-terminal and central putative helix domains and indicate that the residues that define nuclear localization are similar but not identical to the determinants of virion incorporation.

MATERIALS AND METHODS

Generation of wild-type and mutant Vpr expression plasmids

Vpr expression was evaluated using a two-component system based on a recombinant vaccinia virus expressing the T7 polymerase gene (vTF7-3) and a T7-driven Vpr expression vector (Mahalingam *et al.*, 1995a). The Vpr wild-type expression plasmid was generated by PCR amplification of *vpr* of the infectious molecularly cloned HIV-1 strain 89.6 (Collman *et al.*, 1992), using primers that incorporate *Hin*dIII and *Xho*I restriction sites at the 5′ and 3′ ends, respectively. The control plasmid Δvpr was generated similarly except that a proviral clone with stop codons in place of residues 8 and 11 (Balliet *et al.*, 1994) was used as a template. Mutations were introduced by overlap extension PCR as described (Mahalingam *et al.*, 1995a), using wild-type proviral DNA as the template. PCR amplification products were then digested with *Hin*dIII and *Xho*I and cloned into pCDNA3 (Invitrogen, San Diego, CA) between the T7 promoter and the bovine growth hormone polyadenylation signal. Each plasmid was verified by DNA sequencing to ensure the presence of the target mutation and to be certain that no additional mutations were introduced by PCR. Mutations utilized in this study are shown in Fig. 1. Details of the expression plasmids and PCR primers used have been described previously (Mahalingam *et al.*, 1995b).

Cells and transfections

HeLa cells were maintained in DMEM containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) and plated on to poly-L-lysine-coated glass coverslips (eight coverslips in the bottom of 35-mm round tissue culture plates) at a density of 1×10^6 cells per dish. Twenty-four hours later they were infected with vTF7-3 at a multiplicity of infection of 10. One hour later the cells were washed with PBS and refed with serum-free medium (Opti-MEM; Gibco BRL, Gaithersburg, MD). They were then transfected by Lipofectin (Gibco BRL) with Vpr expression plasmids as previously described (Mahalingam *et al.*, 1995b). DNA (3–6 μg) and Lipofectin (10 μl) were suspended in 100 μl each of serum-free medium, mixed together, incubated at room temperature for 15 min, and then added to the cells. Three hours later 0.8 ml of DMEM containing 10% fetal bovine serum was added and the incubation continued overnight.

Immunofluorescent detection of Vpr

Sixteen to 24 hours after transfection the cells were washed with PBS and fixed with methanol at room temperature for 30 min. They were again washed, incubated for 90 min with primary antiserum diluted 1:50 in PBS, washed, and incubated for 90 min with FITC-conjugated affinity-purified F(ab)′2 fragment of goat anti-rabbit IgG (ICN Biochemicals, Costa Mesa, CA) diluted 1:100 in PBS. Coverslips were then counterstained for 5 min with Evans blue (0.02% in PBS; Sigma, St. Louis, MO), washed, and mounted on glass slides using a fade-resistant mounting medium (Citifluor; London, England). All incubations were carried out at 37° in a humidification chamber. The primary antibody was a polyclonal rabbit antiserum raised against a 96-amino-acid synthetic peptide derived from the Vpr sequence of HIV-1 Bru (Mahalingam *et al.*, 1995a; Myers *et al.*, 1993), and preimmune serum from the same animal was used in parallel as a control. For each mutant at least 50 positive cells were analyzed in each of three independent transfection experiments, and slides were interpreted blindly.

Virion incorporation function

The ability of Vpr proteins to undergo Gag-directed virion incorporation was determined as previously described (Mahalingam *et al.*, 1995a). Briefly, vTF7-3-infected HeLa cells were cotransfected with T7-driven expression vectors containing both *gag* and either wild-type or mutant *vpr*. Cultures were incubated in cysteine and methionine-deficient medium and then labeled for 5 hr with ^{35}S-protein labeling mix. Culture supernatant was then collected, clarified by low-speed centrifugation, concentrated using a 30-kDa size-exclusion filter, and immunoprecipitated using the anti-Vpr antiserum. Immunoprecipitated proteins were subjected to SDS–PAGE and analyzed by autoradiography.

RESULTS

Vpr expression system and generation of mutants

To study subcellular localization we utilized the *vpr* gene derived from a macrophage-tropic HIV-1 primary isolate, 89.6, because it is in infection of macrophages that Vpr appears to play its most prominent role in virus replication (Westervelt *et al.*, 1992; Balotta *et al.*, 1993; Hattori *et al.*, 1990; Balliet *et al.*, 1994). In addition, *vpr* deletion significantly restricts 89.6 replication in primary macrophages (Balliet *et al.*, 1994), which suggests that this *vpr* allele is likely to accurately represent a native gene with a full complement of wild-type functions. The DNA and predicted protein sequence of 89.6 *vpr* has

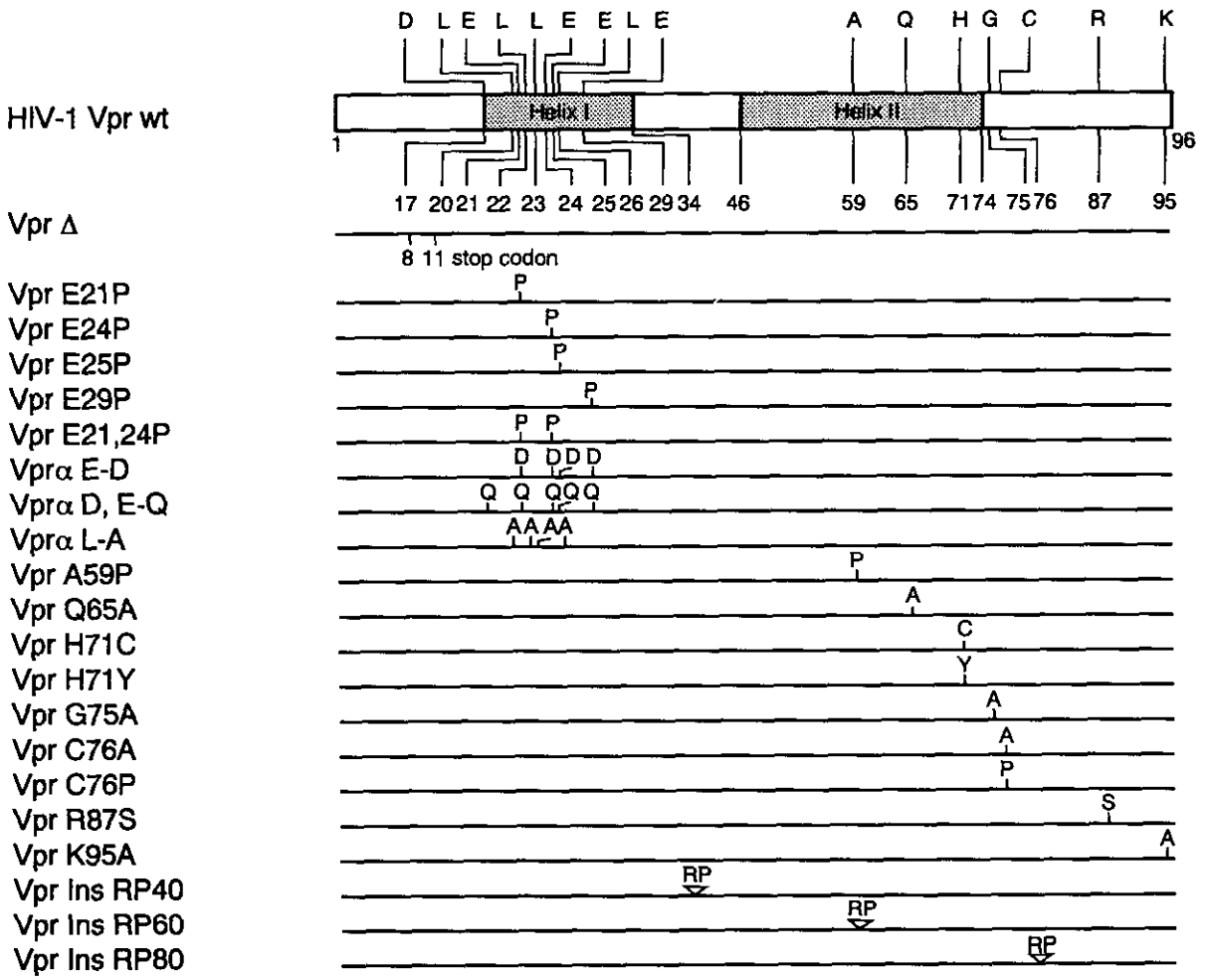

FIG. 1. HIV-1 Vpr mutants utilized in this analysis. Locations of residues targeted for mutagenesis are shown at the top. Shaded areas represent domains predicted to posses an α-helical secondary structure with high probability (residues 17–34) or moderate probability (residues 46–74). The mutant expression plasmids are shown below.

been reported previously (Collman *et al.*, 1992; Mahalingam *et al.*, 1995b).

To address the role of specific residues in intracellular localization, we constructed a panel of mutants that substituted conserved amino acids in specific domains of the protein (Fig. 1). First we tested residues in the N-terminal region (amino acids 17–34), which is strongly predicted to have an amphipathic α-helical secondary structure. Since our previous experiments showed this domain to be critical in Vpr expression, stability, and virion incorporation (Mahalingam *et al.*, 1995a,b), we placed mutations in this region that were designed to disrupt the putative helix. Second, we tested a region spanning amino acids 46 through 74 which also is predicted to form a helical structure, although with a significantly lower hydrophobic moment (data not shown). Third, a pair of highly conserved Gly and Cys residues at positions 75 and 76 were examined. Fourth, we tested the role of conserved charged residues in the basic C-terminal region, since positively charged domains are frequently associated with nuclear localization and because data from other groups has implicated this domain in intracellular localization (Lu *et al.*, 1993). Lastly, we determined the effect of structural disruption at several different positions (after codons 40, 60, and 80) by insertion of two residues (Arg–Pro) designed to affect both charge and structure.

Expression and localization of wild-type Vpr

Vpr localization was examined in HeLa cells that were infected with a recombinant vaccinia virus which expressed T7 polymerase and then transfected with plasmids containing *vpr* under control of the T7 promoter. Vpr was detected by indirect immunoflourescent staining of permeabilized cells, using a polyclonal anti-Vpr rabbit serum. As a control within each experiment we examined cells transfected with a plasmid that was identical to wild-type *vpr* except that it contained stop codons in place of residues 8 and 11 of the open reading frame (Δvpr), as well as mock-transfected cells.

Wild-type HIV-1 Vpr expression was strongly localized

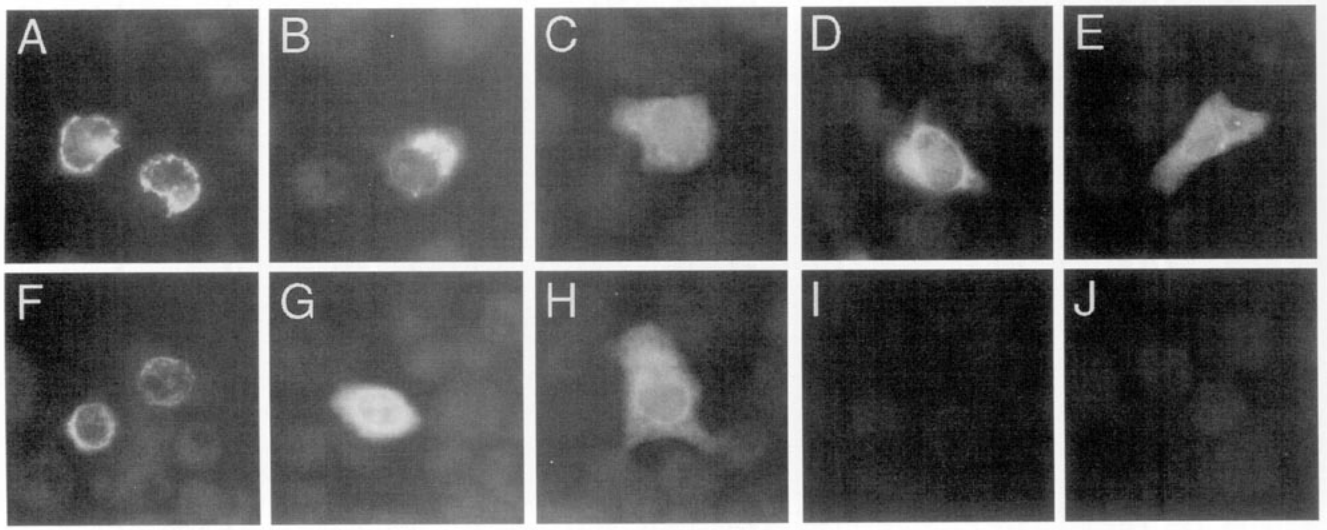

FIG. 2. Cellular expression of wild-type Vpr and the effect of mutations in the N-terminal region. HeLa cells were infected with recombinant vaccinia virus vTF7-3 and 1 hr later transfected with *vpr* expression plasmids under control of the T7 promoter. After overnight incubation the cells were fixed, permeabilized, and stained with anti-Vpr serum as described under Materials and Methods. (A) Wild-type Vpr; (B) E21 → P; (C) E24 → P; (D) E25 → P; (E) E29 → P; (F) αE → D; (G) αD,E → Q; (H) αL → A; (I) Δvpr; and (J) wild-type Vpr stained with preimmune serum. Magnification ×630.

to the nuclear region (Fig. 2A), consistent with prior reports of Vpr subcellular localization (Lu *et al.*, 1993; Zhao *et al.*, 1994a). As has been reported previously (Lu *et al.*, 1993), several patterns were seen. All cells had an intense signal at the rim of the nucleus, suggesting an association with the nuclear membrane, which in some cells had a smooth appearance while others displayed a focal distribution. In addition, diffuse staining of lesser intensity was present throughout the nucleus in most cells. Finally, a small perinuclear focus of staining was also sometimes present. No cytoplasmic expression was seen in cells transfected with wild-type *vpr*. The specificity of this staining was demonstrated by the absence of a signal in cells transfected with the Δvpr plasmid (Fig. 2I) and in mock-transfected cells (data not shown) and by the lack of staining in cells transfected with wild-type plasmid but stained with preimmune serum (Fig. 2J). In addition, immunoprecipitation with this serum revealed the expected 14-kDa band in cells transfected with wild-type *vpr* but not in Δvpr or mock-transfected cells, nor was the band seen with preimmune serum (data not shown).

Mutagenesis of the N-terminal putative α-helical region

The putative N-terminal amphipathic α-helix is located at residues 17 through 34 and contains five acidic amino acids and four hydrophobic leucines (Mahalingam *et al.*, 1995a). This helix structure is highly conserved among HIV-1, HIV-2, and SIV isolates, and the only sequence difference in strain 89.6 as compared to other HIV-1 isolates is an acidic Asp at residue 17 instead of the more common acidic Glu. A homologous structure is also predicted for Vpx of HIV-2 and SIV (Tristem *et al.*, 1992; Kappes *et al.*, 1993). We previously showed that this region is essential for Vpr incorporation into virions (Mahalingam *et al.*, 1995a,b), and we therefore tested the role of this domain in Vpr subcellular localization. Disruption of helices by proline substitution is well described (Horwich *et al.*, 1986; Tacke *et al.*, 1993), and so immunofluorescent patterns were examined for mutants in which each of the four conserved Glu residues were replaced by Pro individually (E21 → P, E24 → P, E25 → P, and E29 → P) or in combination (E21,24 → P). Three additional mutants were generated in which multiple residues were substituted. Vpr αD,E → Q replaced five acidic residues with uncharged Gln residues, αE → D replaced the four acidic Glu residues with conservative acidic Asp substitutions, and αL → A replaced the four bulky nonpolar Leu residues with smaller Ala residues.

Each of the proline substitutions resulted in loss of the highly specific wild-type nuclear pattern (Figs. 2B-E and Table 1). Vpr E24 → P and E29 → P produced diffuse expression throughout the cytoplasm and nucleus, suggesting a lack of compartmentalization, while cytoplasmic expression predominated in E25 → P. Interestingly, mutants E21 → P (Fig. 2B) and E21,24 → P (not shown) had cytoplasmic staining mainly in a region near the nucleus but much larger and more diffuse than the perinuclear foci seen with wild type. Whether this reflects localization in some other specific compartment, such as the golgi, is currently under investigation. Substitution of Ala for the four hydrophobic Leu residues in this region (αL → A) also resulted in diffuse expression (Fig. 2H). When the acidic charge of this domain was altered by substituting uncharged Gln for each of the five acidic

TABLE 1

Effect of Mutagenesis on Vpr Subcellular Localization and Virion Incorporation Functions

Plasmid	Subcellular localization[a]	Virion incorporation[b]
Wild-type	wt	+
Δvpr	None	−
Mock	None	−
E21 → P	Focal cytoplasmic > nuclear	−
E24 → P	Diffuse	−
E25 → P	Cytoplasmic > nuclear	−
E29 → P	Diffuse	−
E21, 24 → P	Focal cytoplasmic > nuclear	−
αE → D	wt	+
αD, E → Q	Cytoplasmic + punctate nuclear	+
αL → A	Diffuse	−
A59 → P	Cytoplasmic + punctate nuclear	−
Q65 → A	wt	+
H71 → Y	Cytoplasmic > nuclear	±
H71 → C	Cytoplasmic > nuclear	+
G75 → A	wt	+
C76 → P	wt	+
C76 → A	wt	+
R87 → S	wt	+
K95 → A	wt	+
RP insert 40	Cytoplasmic + punctate nuclear	−
RP insert 60	Cytoplasmic + punctate nuclear	−
RP insert 80	wt	+

[a] Subcellular pattern of Vpr expression detected by immunofluorescent staining: "wt" indicated wild-type pattern with intense expression at the nuclear membrane, diffuse intranuclear expression, and small perinuclear focal staining.

[b] Incorporation of Vpr into Gag-directed virion-like particles determined by immunoprecipitation of size-selected cell supernatant (Mahalingam *et al.*, 1995a, b); +, Vpr detected; −, no Vpr detected; ±, extremely faint Vpr signal detected.

residues (αD,E → Q) the protein showed intense cytoplasmic expression (Fig. 2G), along with some staining at the nuclear rim. In addition, αD,E → Q also showed strong intranuclear expression, but unlike the diffuse nuclear pattern of wild-type Vpr, this mutant showed large focal intranuclear deposits, the significance of which is not known. In contrast, when the four Glu residues were replaced with charge-conserving Asp, expression was indistinguishable from that of wild type (Fig. 2F). Thus, substitutions that were predicted to disrupt the α-helix impaired Vpr subcellular expression patterns, while multiple changes that conserved charge and structure supported normal localization. Of note, among both the wild type and the mutants tested, there was considerable variation between individual cells in the intensity of fluorescence staining, but there did not appear to be any correlation between the level of intensity and the patterns of distribution.

Effect of substitutions in the central predicted helical region

Between residues 46 and 74 of Vpr is a domain that is also predicted to form an α-helix (data not shown), and three conserved residues within this region were selected for mutagenesis (Fig. 3). Ala-59 is conserved among HIV-1 Vpr sequences but is absent in HIV-2 and SIV Vpr, while Gln-65 and His-71 are conserved in essentially all Vpr proteins (Tristem *et al.*, 1992). Vpr A59 → P showed expression throughout the cytoplasm and the nucleus, with prominent focal intranuclear staining (Fig. 3A) that was similar to that of αD,E → Q, while replacement of His-71 with either Tyr (H71 → Y, Fig. 3C) or Cys (H71 → C, not shown) produced intense cytoplasmic expression. In contrast, substitution of Gln at codon 65 with Ala (Q65 → A) resulted in an immunofluorescence pattern that was similar to that of wild type (Fig. 3B).

Role of the conserved glycine and cysteine residues

Comparison of multiple Vpr sequences indicates that Gly and Cys residues at positions 75 and 76 are universally conserved. In addition, even though Cys is frequently associated with critical structural and functional characteristics, we previously found that Cys-76 was dispensable for Vpr packaging into virions (Mahalingam *et al.*, 1995a). To determine whether these amino acids were essential for subcellular localization, we substituted Ala for the conserved Gly (G75 → A) and both Pro and Ala for Cys (C76 → P and C76 → A). Each mutant showed nuclear expression that was indistinguishable from that of wild type (Figs. 3D–F), indicating that the conserved G75 and C76 are not necessary for subcellular localization. In addition, it shows that the localization impairment resulting from proline substitutions in the α-helix is not a nonspecific result of Pro insertion in the protein, since placement at residue 76 had no effect.

Basic residues of the C-terminal region in nuclear localization

The C-terminal domain of HIV-1 Vpr contains a series of positively charged amino acids. While this domain is less well conserved among SIV *vpr* genes (Tristem *et al.*, 1992), basic regions are typically associated with nuclear localization sequences (Hanover, 1992). We therefore determined the effects of specific positively charged amino acids in this region by substituting two basic residues with uncharged amino acids. The conserved Arg at position 87 was replaced with Ser in mutant R87 → S. Strain 89.6 has a Lys rather than the more common Arg at position 95, and this was replaced with Ala (K95 → A). Both R87 → S and K95 → A were indistinguishable from wild type (Figs. 4A and 4B), indicating that neither of these residues at sites where basic charges are highly conserved is necessary for normal localization. Because previous studies have reported conflicting data on the effect of C-terminal deletions on nuclear localization (Lu *et al.*, 1993; Zhao *et al.*, 1994a), we also constructed two C-terminal deletion mutants by inserting termination codons after residues 76 and 85, respectively (data not

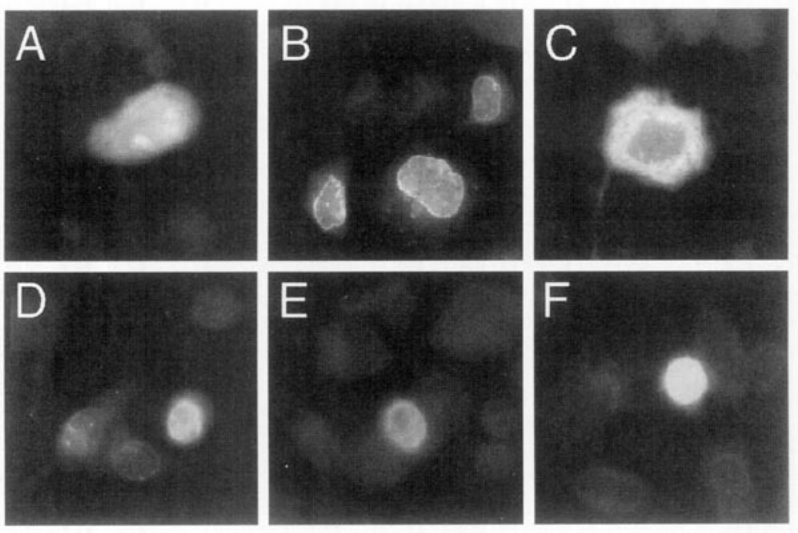

FIG. 3. *Subcellular localization of Vpr proteins with mutations in the central region. vTF7-3-infected HeLa cells were transfected with vpr expression* plasmids and then fixed and stained with anti-Vpr serum as described under Fig. 2 and Materials and Methods. (A) A59 → P; (B) Q65 → A; (C) H71 → Y; (D) G75 → A; (E) C76 → P; and (F) C76 → A. Magnification ×630.

shown). Surprisingly, when transfected into vTF7-3-infected HeLa cells, neither of these two proteins could be detected by immunofluorescence staining. Pulse-chase analysis demonstrated that these C-terminal deletion mutants had markedly decreased stability (data not shown), which likely accounts for our inability to detect them by immunofluorescence staining. Thus, these experiments demonstrate that specific basic residues in the C-terminal region are dispensable for nuclear localization, although they do not clarify the discordant results reported on the effects of C-terminal deletions.

Effect of insertional mutations

We then examined the effects of structural disruption by introducing two amino acids after codons 40, 60, 70, and 80, respectively, of the *vpr* sequence. These residues, Arg–Pro, were selected because they would be likely to introduce a significant charge and structural alterations. Insertion between the two putative helix domains (RP40) or within the second domain (RP60) resulted in aberrant localization (Figs. 4C and 4D), with strong cytoplasmic expression and punctate nuclear staining similar to that of αD,E → Q and A59 → P. Mutant RP70 could not be detected either by immunofluorescence or by immunoprecipitation (data not shown), suggesting that disruption in this region had a dramatic effect on protein expression or stability. In contrast, insertion at residue 80 (RP80) had no effect on Vpr localization (Fig. 4E). These data provide additional support for a role for the two putative helical domains in subcellular localization and also suggest that the structure of the region between them may be important as well. In addition, they further strengthen the view that the C-terminal region is not a major determinant of subcellular localization function.

Relationship between subcellular localization and virion incorporation functions

We previously identified mutations in Vpr that impaired Gag-directed virion incorporation (Mahalingam *et al.*, 1995a,b). To determine the relationship between domains that underlie Vpr's two localization functions, we

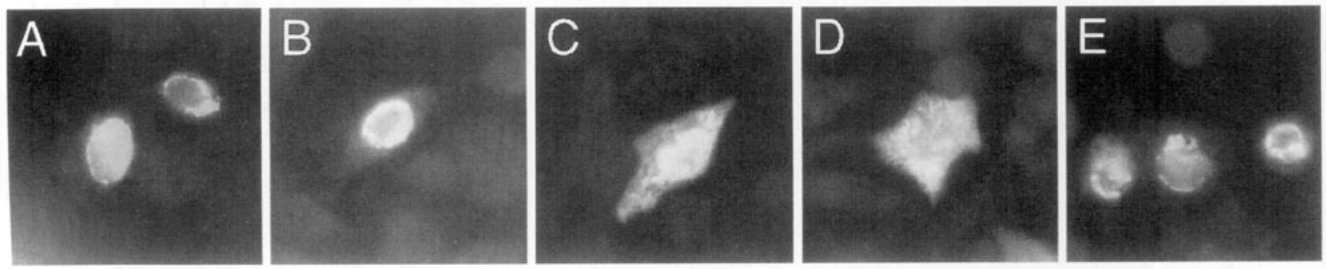

FIG. 4. Subcellular localization of Vpr proteins with C-terminal mutations or two-residue insertions. vTF7-3-infected HeLa cells were transfected with vpr expression plasmids and then fixed and stained with anti-Vpr serum as described under Fig. 2 and Materials and Methods. (A) R87 → S; *(B) K95 → A; (C) RP-40 insert; (D) RP-60 insert; and (E) RP-80 insert. Magnification* ×630.

compared the results of these subcellular localization experiments with those of virion incorporation (Table 1). To evaluate virion incorporation, vTF7-3-infected HeLa cells were cotransfected with both a *gag* expression plasmid and either a wild-type or a mutant *vpr* expression plasmid (Mahalingam *et al.*, 1995a). Virion-like particles were separated by size-exclusion filtration of supernatant and then immunoprecipitated using an anti-Vpr antiserum. No Vpr was detected in either the whole or the size-selected supernatant without *gag* cotransfection, indicating that its export was dependent on Gag-directed particle incorporation (Mahalingam *et al.*, 1995a). Table 1 shows that there was a close relationship between the effect of specific mutations on subcellular distribution and virion incorporation. However, Vpr αD,E → Q was incorporated efficiently into Gag-directed particles despite a subcellular expression pattern that was distinctly different from that of wild type. Similarly, mutant H71 → C showed impaired localization but incorporated into particles. Thus, the structural determinants of nuclear localization are closely related to, but not identical with, those that underlie virion incorporation.

DISCUSSION

HIV-1 Vpr has two distinct localization properties that direct it to the cell nucleus (Lu *et al.*, 1993; Zhao *et al.*, 1994a) and, in association with Gag, directed it into the assembling viral particle (Lavallee *et al.*, 1994; Lu *et al.*, 1993; Paxton *et al.*, 1993; Mahalingam *et al.*, 1995a). Vpr incorporation into virions depends on the p6 region of Gag (Lavallee *et al.*, 1994; Lu *et al.*, 1993; Paxton *et al.*, 1993), and we recently identified domains of Vpr that determine its virion incorporation function (Mahalingam *et al.*, 1995a,b). In this report we identified regions of Vpr that determine its nuclear localization function. This is the first study to undertake extensive mapping of the HIV-1 Vpr determinants of subcellular localization and to define the relationship between the structural elements responsible for its two localization functions.

A striking finding of these experiments was that the mutations in several widely separated regions of Vpr affected localization. One critical region was the N-terminal putative helical domain, in which several mutations suggested that the predicted helical structure was essential. A second region with the potential for helical structure in the central portion of the protein was also important for localization. Since intraprotein interactions between helix domains may determine protein conformation, it is possible that interactions between the two putative helical domains of Vpr are necessary for maintenance of the tertiary structure required for function. Consistent with this, insertion of the Arg–Pro dipeptide in between the two putative helical regions (RP40) also impaired localization. The mechanism by which Vpr translocates to the nucleus, however, and the means by which these residues mediate this function are unknown. The sequences identified do not contain a nuclear localization motif that would be expected to be directly responsible for nuclear targeting (Hanover, 1992). Helix structures are known to support protein–protein interactions (Tacke *et al.*, 1993), and it thus may be through association with cellular factor(s) that Vpr translocates to the nuclear region. Consistent with this, recent observations suggest that Vpr can associate with cellular factors (Refaeli *et al.*, 1995; Zhao *et al.*, 1994a), including a putative glucocorticoid receptor complex with which it may translocate to the nucleus (Refaeli *et al.*, 1995).

In contrast to the putative helical domains, the conserved Gly–Cys (75, 76) motif was dispensable for Vpr localization. Neither of these amino acids was essential for Gag-directed virion incorporation either (Mahalingam *et al.*, 1995a, and Table 1) and so the essential function(s) for these residues, which are found in virtually all primate immunodeficiency Vpr sequences, remains unknown. Of note, these residues are also present in Vpx, which does not localize in the same pattern as Vpr (Kappes *et al.*, 1993; Wu *et al.*, 1994). Another target for investigation has been the basic C-terminal region, since positively charged residues are typically associated with nuclear localization (Hanover, 1992). One previous study reported that deletion of the C-terminal 19 residues disrupted subcellular distribution (Lu *et al.*, 1993), but another reported that deletion of residues 78–87 or 88–96 or mutation of basic residues in the region had no effect (Zhao *et al.*, 1994a). Our results are concordant with the view that this domain is not critical for subcellular localization and are consistent with the observation that SIV Vpr localizes in a nuclear pattern similar to HIV-1 Vpr even though it does not possess this basic C-terminal motif (Zhao *et al.*, 1994a; Tristem *et al.*, 1992).

These studies map determinants of localization function since they clearly discriminate between wild-type nuclear and aberrant cellular localization, but they do not provide quantitation of protein levels within each compartment nor do they give quantitative information about expression levels overall. Nevertheless, it is unlikely that effects on localization resulted from altered expression levels or protein stability, as there did not appear to be a relationship between immunofluorescent staining intensity and localization patterns. In addition, we recently found that some mutations in the N-terminal putative helix affected protein stability as determined by pulse-chase analysis (Mahalingam *et al.*, 1995b), but the current results on subcellular distribution did not correlate with altered stability. For example, αD,E → Q had no effect on stability but disrupted subcellular localization, while another mutation showed markedly decreased stability but had normal localization (D17 → P; data not shown). Finally, the C-terminal truncation mutants were extremely unstable, yet immunofluorescent analysis

showed no detectable staining rather than aberrant localization.

The close correlation between the effects of mutations on subcellular localization and those on virion incorporation suggests that these two functions may be supported by similar mechanisms. However, these functions were discordant in two mutants that localized mainly to the cytoplasm but incorporated into virion particles when expressed with Gag. Chou–Fassman analysis of one of these (αD,E → Q) indicated that it does have the capacity to form a helix but with a much lower predictive value than that of the native sequence (Mahalingam *et al.,* 1995b, and data not shown), and it is therefore possible that this mutant may have a partially intact structure that is able to support some but not all Vpr functions defined by this region. Alternatively, it is possible that nuclear localization and virion incorporation are subserved by distinct elements of Vpr that have yet to be defined and that mutagenesis of regions identified in this analysis disrupts overall protein structure and interferes with functions of other regions. Furthermore, Vpr forms oligomers (Paxton *et al.,* 1993; Bogerd *et al.,* 1993; Zhao *et al.,* 1994b) and the N-terminal domain was recently reported to mediate oligomerization of bacterially produced Vpr (Zhao *et al.,* 1994b), but whether oligomerization in eukaryotic cells is necessary for Vpr localization or other functions remains to be determined.

Thus, our structure–function analysis suggests that two localization functions of Vpr, nuclear localization and virion incorporation, are mediated by similar regions, including the N-terminal putative helix and residues in the central domain with potential for helical structure. Further investigation will be necessary to define whether this reflects common pathways involved in localization. Several additional conserved regions of the protein, notable the GC dipeptide motif and C-terminal basic domain, have yet to be assigned critical roles in Vpr's activity.

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